Standard Operating Procedure
for
Hazardous Chemicals

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Building and rooms: Davison Life Sciences Building, Lab B310

Chemical(s)	ABsolute™ QPCR SYBR Green Mix Plus ROX, SuperScript II Reverse Transcriptase, RNase inhibitor, Turbo DNA-*free*™, Agarose, Ethidium bromide (EtBr), Dithiothreitol (DTT).
Process	**SYBR Green QPCR**
Specific Hazards *referred to MSDSs for more detailed information*	EtBr (Ethidium bromide): Potent mutagen, moderately toxic. DTT (Dithiothreitol): may be toxic to central nervous system (CNS). Repeated or prolonged exposure to the substance can produce target organs damage.
Personal protective equipment	Must wear 3-5 mil nitrile gloves. Chemical safety goggles and lab coat should be worn when splash potential exist
Engineering/ventilation controls	Emergency shower and eyewash accessible.
Special handling procedures and storage requirements	Store EtBr away from oxidizers and flammable reagents. Store all stock kits @ -80°C, working kit of SYBR Green Mix Kit @ 4°C, Reverse Transciptase, RNase inhibitor, and Turbo DNA*free* @ -20°C, all the other buffers on bench top.
Spill and accident procedures *for hazardous* *chemicals only*	Skin exposure: Rinse affected skin with plenty of water while removing contaminated clothing and shoes. Rinse for at least 15 minutes. Seek medical attention. Eye exposure: Wash eyes for at least 15 minutes, lifting the upper and lower eyelids. Seek medical attention immediately.
	Small (< 1L): If in solution, absorb freestanding liquid. Use ultraviolet light to locate spill, absorb with spill pads and transfer absorbed material to a closed container. Label and date as hazardous waste for disposal. Notify PIs.
	Large (> 1L): Evacuate the room, notify PIs and call 2-5801 to request emergency assistance from Environmental Safety Division
Waste disposal	EtBr gels: gels with <0.1% EtBr can be placed in regular trash; gels with EtBr >0.1% should be collected in a sturdy plastic bag, placed in a cardboard box, labeled and disposed of as non-RCRA hazardous waste via the ESD (Environmental Safety Division).
Special approval	No special authorization needed after SOP training & reading MSDSs.
Prepared by	Name/date: C-J Tsai, 8/31/09, revised 9/14/2018, 7/20/2023

SYBR Green QPCR Standard Operating Procedures

Chemicals and Reagents

ABsolute Blue QPCR SYBR Green Mix with ROX (Thermofisher AB4167A, 400 Rxn/Pk, 25µL/Rxn) or

Agilent Brilliant III UF MM SYBR QPCR Low ROX (Agilent 600892)

QPCR Plastics (non-skirted 96-well plate or 8-well strip tubes) must be **optically clear**.

Anchored Oligo dT primer: 5'-TTTTTTTTTTTTTTTTTTTTVN (can be custom synthesized)

Random hexamers or decamers (custom synthesized or from the RT kit)

High-capacity cDNA Reverse Transcription Kit (ThermoFisher Applied Biosystems 4368814)

RNase inhibitor is included in the kit. ThermoFisher Ambion N808-0119 (20 U/µL) can also be used.

RNA loading dye: Add 1 µL of EtBr (10 mg/ml) to 500 µL of DNA 6X loading dye (brand new). Dilute 2X, aliqout and store separately from other DNA reagents at -80°C.

RNase-free water: DO NOT use DEPC-treated water, as incomplete removal of DEPC may interfere with downstream applications. Best source of RNase-free water is directly from the water purification system, after letting it run for ~30 sec, into a sterile Falcon tube. DO NOT use water from a storage Carboy. You may autoclave the water, but it's optional.

RNA storage, handling and QC

RNA should be free of genomic DNA contamination. The original stock should NOT be diluted for long-term storage. To avoid repeated freeze-and-thaw cycles, it is advised that you prepare two 'working' aliquots of each RNA sample based on NanoDrop. Working stock RNA concentration for the whole group of samples (from the same experiment) is usually that of the lowest-yielding RNA sample of the group, and ideally should have a concentration >50 ng/µl (20 ng/µl also worked for difficult samples). Samples with extremely low RNA concentrations should be repeated or RNA concentrated by speed vac.

Perform agarose gel electrophoresis by loading an equal amount of RNA (100-200 ng per lane) using **RNA loading dye**, and adjust the concentration estimates based on band intensities.

The electrophoresis tank should be pre-treated with 2% AbSolve (shake well before use) in ddH2O for 1h to overnight, and thoroughly rinsed with ddH2O (2-3X) before use. The AbSolve solution can also be used for other cleaning purposes within a few days (wiping off the pipet shaft or your work area).

Disposable plasticware such as centrifuge tubes and pipette tips are usually free of RNase contamination and can be used as is (from sterile unopened packages) or simply autoclaved without any pretreatment. However, it is advised that opened bags of plasticware be tightly closed and sequestered from general lab circulation if they are to be used for future RNA work.

Gloves must be worn when working with RNA. Touching freezer/refrigerator handles, door knobs, or any surfaces that are not RNase-free will contaminate gloves. It is good to wipe your gloves off with a Kimwipe of 70% ethanol if you contaminate them at the same time you are handling RNA.

RT reaction

Either anchored oligo dT primers or random hexamers/decamers can be used for cDNA synthesis. Oligo dT primers provide better specificity, but may be biased against the 5'-ends. Random primers generate the most diverse cDNA pool with higher yields. For some challenging targets (long or secondary structure-rich), it may be advantageous or necessary to use a mixture of oligo dT and random primers. In general, we use both for qRT-PCR, and oligo-dT only if cloning of full-length cDNA is desired. Discuss with CJ if you are unsure. **Make master mixes and avoid pipetting anything less than 0.5 μL.**

1. Prepare 2X master mix (MM) with 10% extra, rounding to the nearest integer.
   Final reaction = 10 μL each, hence the 2X MM should be 5 μL per reaction.

	2X MM (μL) per reaction	Total x ____ (μL)
10X RT buffer	1	
RNase inhibitor	0.4	
25X (100 mM) dNTPs	0.4	
10X random primers	0.5	
Anchored Oligo dT primers, 5 μM	0.5	
MultiScribe™ Reverse Transcriptase	0.5	
RNase-free water	1.7	
Final volume	to 5 μL	

2. Mix gently by pipetting up and down. Add 5 μL of 2X MM to 5 μL of total RNA, and mix gently.
   Depending on the purpose, 200 -1000 ng of total RNA should be sufficient.

3. Incubate in a thermal cycler with the following program:

   25°C/10 min, 37°C/60 min*, 85°C/5 min, 15°C/hold
   * the reaction time can be extended to 2 hour to increase yield.

4. Store cDNA at -20°C until use.

QPCR reaction

Replication: Perform all reactions with 2 technical replicates and 3 biological replicates. ***Technical replicates must be done together in the same plate***

Housekeeping controls: Use 2 housekeeping genes for comparison between treatments (e.g., same tissue type); Use 3 housekeeping genes for comparison across multiple tissues

Negative controls: For each primer pair and sample type, perform "no-template" and "no-RT" controls to check for non-specific amplification and/or genomic DNA contamination etc.

Primers: Gene-specific primers should target the 3'-UTR regions, or span exon-intron junction, for amplicons of 150-300 bp.

Template optimization: Optimum template amount should be determined using 0.1, 0.5, 1, 5, and 10 ng cDNA for each primer pair to confirm linearity of amplification (if samples are limited, use only 0.1, 1 and 10). We found **1-5 ng** (mid-point) to be suitable for most target genes we have tested.

For simplicity, **cDNA concentration** is determined based on input RNA amount used in RT. For reactions with 200 ng total RNA, the cDNA yield is equivalent to 20 ng/μL. Dilute an aliquot to 1 ng/μL.

ABsolute Blue QPCR SYBR Green Mix with ROX (Thermofisher AB4167A)

Formula for a single reaction (10 μL volume, from 2 master mixes plus cDNA):

Forward primer (1 μM)	0.7 μL (equivalent to 70 nM final concentration)
Reverse primer (1 μM)	0.7 μL
cDNA	2 μL (equivalent to 1-10 ng of total RNA)
2X SYBR master mix	5 μL
Reference ROX dye (1:500)	0.15 μL
ddH_2O	1.45 μL

*you can premix the forward and reverse primers into one tube to reduce pipetting

1. Prepare 2 **master mixes:** (required volume + 10% extra, rounding to the nearest integer)

1.1. Primer mix:	Forward primer	per rxn: 0.7 μL x ___	= total: _____
(2 μL/rxn)	Reverse primer	0.7 μL x ___	_____
	ddH_2O	0.6 μL x ___	_____
1.2. SYBR mix:	2X SYBR	per rxn: 5 μL x ___	= total: _____
(6 μL/rxn)	ROX dye	0.15 μL x ___	_____
	ddH_2O	0.85 μL x ___	_____

2. Add **Primer mix** and **cDNA mix** to each reaction tube/well. Under dim light, add **SYBR mix** to the wells and mix gently.

3. Real-time PCR reaction is performed using Agilent AriaMx with the following settings: 15 min at 95°C followed by 40 cycles of 95°C/15s, 55-60°C/30s (*depending on primer Tm's*), and 72°C/30s.

Agilent Brilliant III UF MM SYBR QPCR Low ROX (Agilent 600892)

Formula for a single reaction (10 μL per reaction). We have also scaled down to 5-8 μL successfully.

Forward primer (5 μM)	0.25 μL (equivalent to 125 nM final concentration)
Reverse primer (5 μM)	0.25 μL
cDNA	2 μL (equivalent to 1-10 ng of total RNA)
2X SYBR master mix with ROX	5 μL
ddH_2O	2.5 μL

1. Prepare **primer mixes:** (required volume + 10% extra, rounding to the nearest integer)

	Forward primer	0.25 μL x ___	= total: _____
(3 μL/rxn)	Reverse primer	0.25 μL x ___	_____
	ddH_2O	2.50 μL x ___	_____

2. Add **Primer mix** and **cDNA** to each reaction tube/well. Under dim light, add **SYBR mix** to the wells and mix gently.

3. Real-time PCR reaction is performed using Agilent AriaMx with the following settings: 3 min at 95°C followed by 40 cycles of 95°C/5s, 60°C/5-10s (depending on primer Tm's and amplicon length).

DO NOT mix and match. The ULTRA FAST program can only be used with Agilent UF SYBR kit